NITROGEN FIXATION IN LEGUMINOUS PLANTS. IV.

THE INFLUENCE OF REACTION ON THE FORMATION OF ROOT NODULES IN MEDICAGO AND TRIFOLIUM.

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Introduction.

In the cereal-growing as well as the pastoral regions of Australia the nitrogen supply of the crops and herbage depends largely on symbiotic nitrogen fixation by clovers and medics, wild as well as cultivated (Donald, 1941). Since the soil reaction varies widely, from moderately acid in many wheat districts of New South Wales to strongly alkaline in the Mallee soils, a detailed study of this factor in relation to symbiotic nitrogen fixation becomes a problem of high agricultural importance, but it has so far received comparatively little attention. It is generally assumed that a soil reaction near neutrality is the most favourable for nitrogen fixation by legumes, but the degrees of variation that exist in this respect, as well as the interaction between hydrogen ion concentration and other factors regulating plant growth, are still incompletely known (Fred *et al.*, 1932; Wilson, 1940). Apart from indirect effects (on availability of mineral nutrients, production of ammonia and nitrate, etc.), soil reaction may influence the symbiotic nitrogen fixation in several ways, firstly through its effect on survival and multiplication of the root-nodule bacteria in the soil, secondly by affecting the ability of these bacteria to invade the plant roots (a point of obvious significance in relation to artificial seed inoculation), and thirdly through its effect on the metabolism of the host plant, including the bacterial tissue of the nodules, which is the actual seat of the process of nitrogen fixation.

The influence of the hydrogen ion concentration on the *Rhizobium* population of the soil* was first studied in some detail by Bryan (1923*b*), who found that *Rh. Meliloti*, when grown in sterile soil, was killed at pH 4·9–5·1, and *Rh. Trifolii* at pH 4·5–4·9. Wilson (1926*a*, 1926*b*) stated that *Rh. Trifolii* and especially *Rh. Meliloti* were only sparsely represented in field soil of pH 5·4–5·8, and that artificial inoculation greatly stimulated root-nodule formation on lucerne and clover in such soil. In agreement herewith, Bjälfve (1934) found that inoculation of red clover was very beneficial in soil of pH 5·0, but much less so in the same soil limed to pH 6·2. Peterson and Goodding (1941) found a high positive correlation between soil pH, ranging from 5·0 to 8·6, and percentage of soil samples harbouring *Rh. Meliloti*. Almon and Baldwin (1933) mention that *Rh. Trifolii* tends to change into an aberrant, non-infective type by cultivation in acid soil (pH not stated). The alkaline limit for survival of rhizobia in soil does not appear to have been determined.

The influence of reaction on formation of root nodules and subsequent plant growth has been investigated somewhat more fully. Bryan (1923*a*) observed that nodules were formed on red clover, alsike clover and lucerne in sand culture within a pH-interval extending between pH 4–5 and pH 9–10. The highest numbers of nodules and greatest dry weight of plants were produced at pH 7–8. Powers (1927) found that lucerne and alsike clover grew best in water culture at pH 6·0, and that nodules were most numerous at pH 5·8–7·0 but very few at pH 4·8. Virtanen (1928) grew white, red and alsike clover

* Here and in the following, the investigations referring to *Medicago* and *Trifolium* are chiefly considered.

in sand at different degrees of acidity, and found that nitrogen fixation, as measured by growth of inoculated plants in sand without nitrogenous fertilizer, was practically nil at pH 4·5, but increased steeply with increasing pH up to 6·0, which was the highest pH-value studied. Growth with combined nitrogen showed a similar trend but extended over a wider pH-range. Janssen (1929) found somewhat similar results with red and subterranean clover; in soil, the latter species appeared to grow equally well at pH 4·9 and 7·0, and nodules were formed at pH 4·0–4·4. Sewell and Gainey (1930) stated that lucerne grew almost equally well at pH 4·5 and 7·0 when provided with combined nitrogen, but that in nitrogen-free sand, few nodules were formed at pH 6·0 and still fewer at pH 4·0. In natural soil the nodule formation seemed to depend more on the supply of calcium and phosphate than on the reaction; this agrees with the important observation of Albrecht (cit. after Wilson, 1940) that the pH-interval for nodule formation in soybeans is influenced by the supply of assimilable calcium. For this reason many of the reported beneficial effects of lime on leguminous crops (see Fred *et al.*, 1932) may be due to calcium as well as to neutralization of soil acidity. Other investigators have only used natural soil as growth medium. In such experiments, Joffe (1920) found that lucerne formed nodules sporadically at pH 3·8–4·3, and that growth was fairly uniform from pH 4·5 to pH 7·0. More accurate experiments on lucerne and *Medicago lupulina* were made by Olsen (1925), who also found nodules formed, although sparsely, at pH 4·0; crop yield was highest at pH 6·5–7·5. Experiments by Watenpaugh (1936) on lucerne and Hyland (1938) on clovers and *Melilotus* have given somewhat similar results. Peterson and Goodding (1941) found that lucerne formed nodules readily in soil of pH 5·1, if inoculated with rhizobia. In such experiments with natural soil it is mostly impossible to distinguish between nitrogen fixation and uptake of combined nitrogen, so that a true picture of the effectiveness of the nodules at different pH is not obtained. Moreover, a very important factor (and this may apply to sand and soil cultures alike) appears to be the means by which the reaction is altered. Bjälfve (1934) pointed out that acidity produced by mineral acids, such as in the experiments of Joffe (1920), Virtanen (1928) and Janssen (1929), is likely to be far more injurious to the plants than the natural soil acidity, and contrary to Virtanen, he found that red clover, if suitably inoculated, grew and formed nodules almost equally well in soil of pH 5·0 and 6·2, the latter reaction being produced by addition of lime.

In the present series of investigations an attempt is made to distinguish between two chief aspects of the problem of hydrogen ion concentration in relation to symbiotic nitrogen fixation: firstly, its effect on the ability of the rhizobia to infect the plant roots and thus to establish nodule formation, and secondly its influence on the ability of the nodules to provide the plant with nitrogen, i.e., the process of symbiotic nitrogen fixation itself.

Experimental.

Agar cultures.—As a first approach to the problem, plants were grown aseptically in agar. By this method, which permits full bacteriological control, it should be possible to detect differences that might exist in the pH-tolerance of host plants as well as different strains of rhizobia. Three species of *Medicago* (*sativa*, *minima*, and *hispida* var. *denticulata*) and four of *Trifolium* (*repens*, *subterraneum*, *pratense*, and *glomeratum*) were tested. The rhizobia included 8 strains of *Rh. Meliloti* and 8 of *Rh. Trifolii*, representing both rapidly and slowly growing strains of both species. The agar medium had the following composition: KH_2PO_4 0·05%, Na_2HPO_4 0·05%, $MgSO_4$ 0·02%, $CaCl_2$ 0·025%, $FeCl_3$ 0·01% and agar 0·6%. The reaction was adjusted by means of H_2SO_4 and NaOH to the desired pH-values, allowance being made for the change in reaction that took place during sterilization, which was effected by steaming in the case of medium of pH 5 and less, otherwise by autoclaving. Disinfected seeds were sown in big test-tubes with 20 ml. medium, and 2 or 3 seedlings were left per tube after germination. When the first true leaf had appeared (usually after 7 to 10 days) the plants were inoculated from young (3–5 days) agar cultures of the rhizobia; care was taken to deposit the inoculum on the surface of the medium immediately adjacent to the seedling. The cultures were placed in a greenhouse and kept under observation for at least 6 weeks. If nodules were formed at all, they usually became visible within one to

two weeks after inoculation; cultures that showed nodule-bearing plants were further observed for about two months to see if there was any stimulation of growth in comparison with uninoculated plants. The results based on some 300 cultures in agar of pH 4·0 to 6·0 are summarized in Table 1; numerous other tests showed constant nodule formation at pH-values above 6.

TABLE 1.

Influence of Reaction on Nodule Formation in Agar Cultures.

Plant.	Nodules produced in Agar of pH :						
	4·0–4·1	4·2–4·3	4·4–4·5	4·7–4·8	5·1–5·2	5·3–5·4	5·9–6·0
Medicago sativa		−	−	−*	±	±	+
M. denticulata			−	−	−	±	+
M. minima			−	−	−	−	±
Trifolium repens	−	±	±	+	+	+	+
T. glomeratum	−	±	±	+		+	+
T. subterraneum	−	±	±	+	+	+	+
T. pratense	−	−	±	+	+	+	+

* One plant showed a single nodule *above* the surface of the agar.

A characteristic difference is seen between the two genera of host plants. In *Medicago* the pH-limit for nodule formation coincides very nearly with the limit for growth of *Rh. Meliloti* in pure culture at pH 5·3–5·5 (Jensen, 1942). At this reaction the formation of nodules in *M. sativa* and *M. denticulata* is quite irregular; for instance, only 6 out of 23 cultures of lucerne at pH 5·3–5·4 showed nodules. At still higher acidity, nodule formation in lucerne is quite exceptional (one positive among 11 cultures at pH 5·14). *M. minima* seems even more sensitive; 12 cultures at pH 5·3–5·4 were all negative. In *Trifolium*, on the other hand, there is quite a wide interval between the limits for growth of *Rh. Trifolii* in pure culture (pH 5·1–5·3) and for nodule formation which took place constantly at pH 4·7–4·8, frequently at pH 4·5, and even at pH 4·2; at the last reaction, indeed, the plants remained as small as the uninoculated ones, showed signs of nitrogen starvation, and seemed upon the whole to derive no benefit from the nodules. The cause of this difference between the pH-limits is not quite clear. The possibility exists that pH-values too low for actual growth of the rhizobia might be less rapidly bactericidal to *Rh. Trifolii* than to *Rh. Meliloti*, so that the former might find time to enter the root tissues where the reaction is but slightly influenced by the medium. This hypothesis, however, could not be verified. When well-buffered sucrose-yeast extract solutions of varying reaction were heavily inoculated with rhizobia and the survival of these tested by transfer to agar slopes, *Rh. Meliloti* was found to be killed within one to three days at pH 4·8 to 5·2, and *Rh. Trifolii* at pH 4·4 to 4·8. The bacteriostatic pH-values are thus also rapidly bactericidal; one strain of *Rh. Trifolii*, indeed, remained alive for three weeks at pH-values as low as 3·7, but this strain was no different from the rest in its nodule-forming capacity. Neither did the difference seem to be due to any ability of the clover roots to modify the reaction of the medium; seedlings of clovers as well as medics were not found to alter the reaction of the agar by more than about 0·2 pH-units during the first few weeks, and then usually in the acid direction. With increasing growth the medium always became strongly acid, as shown below.

A definite limit of alkalinity for nodule formation was not determined, but it was observed that clover as well as lucerne developed nodules regularly in $CaCO_3$-buffered agar of pH 7·6 to 8·7, although this range of reaction inhibits growth of *Rh. Trifolii* in pure culture (Jensen, 1942).

A few quantitative nitrogen-fixation experiments in agar of different pH were also made.* One-pint milk bottles were used as culture vessels, with 125 ml. agar-medium, and 4 seedlings of lucerne or 6 of white clover. Other details as well as the results are seen in Table 2. Nodules were formed by both plants at all reactions, and the type of

* All nitrogen determinations were made by the Kjeldahl method, using selenium as a catalyst; pH-determinations were made by the glass electrode method.

TABLE 2.

Influence of Reaction on Nitrogen Fixation in Agar Culture.

Plant.	Repl. No.	pH of Medium.		Gain of N per Culture, mgm.*	
		Initial.	Final.	Mean.	Standard Deviation.
	4	5·5	4·0–4·5	2·88	±1·60
Lucerne	4	6·3	5·4–5·9	3·70	±0·41
(140 days).	4	7·4	5·3–6·1	2·73	±0·69
	3	4·4	3·6–3·7	3·90	±0·33
White Clover	3	5·4	3·8–3·9	3·97	±0·25
(104 days).	3	6·4	4·3–4·4	3·91	±0·12
	3	6·7†	7·5–7·7	2·06	±0·41

* After subtraction of the mean N-content of 3 uninoculated control cultures (lucerne 1·19 mgm., clover 0·76 mgm.).

† 0·2% calcium carbonate was added to the medium in this series.

nodulation as well as the general appearance of the plants showed no visible difference, except that the clover plants in agar with calcium carbonate remained considerably smaller and fixed significantly less nitrogen than the rest; this phenomenon may be due to inhibited assimilation of phosphate but hardly of iron, since the plants showed no signs of chlorosis. Otherwise the nitrogen fixation is not significantly influenced by the initial pH, even though the gain by lucerne in the most acid medium is very irregular. Apart from the series with calcium carbonate, the pH-values of the medium are seen to fall very markedly, often to a level far below the limit for nodule formation. Since the initial pH of the most acid media was only slightly above this limit, it appears that in these cultures the plants must have fixed most of their nitrogen at a reaction entirely prohibitive to nodule formation. Another experiment was performed in order to get a more clear idea of this. Lucerne was grown in agar of pH 5·6–5·7, and gains of nitrogen and changes in reaction were determined at successive stages of growth; in the first experimental series, two seedlings were grown in test-tubes with 40 ml. medium, and in the second, five seedlings in pint bottles with 120 ml. medium. Four replicate cultures were analysed each time. The results are seen in Table 3.

TABLE 3.

Nitrogen Fixation and Change of Reaction in Agar Cultures of Lucerne.

Series.	pH.	Total N per Culture, mgm.	
		Mean.	S.D.
1. Test-tubes. Initial pH. ..	5·70		
After 42 days	5·55–5·65	0·56	±0·036
,, 80 ,, ...	5·04–5·36	0·69	±0·088
,, 105 ,, ...	4·82–5·08	1·15	±0·20
,, 130 ,, ...	4·28–4·60	2·12	±0·74
Controls, uninoculated	5·50–5·68	0·53	±0·048
2. Bottles. Initial pH.	5·62		
After 40 days	5·71–5·78	1·57	±0·084
,, 82 ,, ...	5·22–5·38	3·34	±0·52
,, 148 ,, ...	4·50–4·72	7·79	±0·38
Controls, uninoculated	5·52–5·71	1·45	±0·18

In the first series practically all, and in the second series some 70 per cent., of the gain of nitrogen has taken place after the time when the pH of the medium has reached the critical level for nodule formation (pH 5·2–5·4); nodules began to appear during the first few weeks in both series. This simple experiment thus shows clearly that the nitrogen-fixing function of already formed nodules does not cease at a reaction too acid for the formation of new nodules. Determination of the rate at which the nodules fix nitrogen at different reactions must be left for more elaborate experiments. The agar

culture method is less satisfactory for such experiments, owing to the constant tendency of the medium to become acid and the slow rate of growth under these highly artificial conditions. Some further experiments on nodule formation at different reaction were therefore performed in more natural media.

Sand cultures.—These experiments were made under greenhouse conditions, using glazed earthenware pots of 6 in. diameter and holding about 3 kgm. sand. In the first experiment an attempt was made to adjust an almost nitrogen-free river sand of pH 7·3 to pH-values of 6, 5 and 4 by increasing additions of sulphuric acid in amounts calculated from a titration curve. In addition, each pot was given 600 ml. of a nutrient solution containing K_2HPO_4 0·2%, $MgSO_4$ 0·05%, $CaSO_4$ 0·05% and $FeCl_3$ 0·02%, and adjusted to the corresponding pH-value. Control of the reaction, however, proved very difficult; the pots in each series often varied a whole pH-unit from each other, and in the most acid range the pH-values were generally much higher than calculated.

Fairly constant reaction was obtained when the pots had been left standing for about 7 months and had received each 2 gm. $CaHPO_4$ plus sufficient phosphoric acid to change this into $Ca(H_2PO_4)_2$; the pots representing the most alkaline reaction were also given each 0·2 gm. $CaCO_3$. On 9th April, 1941, lucerne and subterranean clover were sown in the pots; the seed was inoculated with effective strains of the corresponding rhizobia. The variety of lucerne used in this and all other experiments was "Giant Upright"; the variety of subterranean clover in this test was the late-flowering "Tallarook"; in the other tests the mid-season variety "Mount Barker" was used. After germination the seedlings were thinned to 20 of lucerne and 12 of clover per pot. The numbers of replicate pots and the pH-values of the sand at the time of sowing were as follows:

pH	7·4-7·7	6·2-6·6	6·0-6·4	5·2-5·8
Replicates of lucerne ..	4	5	4	5
Replicates of clover ..	4	4	5	4

The lucerne was harvested after 105 and the clover after 112 days. Dry weight and nitrogen content of tops and roots were determined, and a number of plants was examined for nodule formation by counting and removing all nodules from the carefully washed roots, weighing them after superficial drying with blotting-paper, and finally determining nitrogen in the dry nodule substance. Allowance was made for the dry matter and nitrogen in the excised nodules when calculating the weights and N-percentages of the roots. The results are seen in Tables 4 and 5.

The yield of lucerne is seen to fall steeply with falling pH, and is extremely small at pH 5·6–5·8, where the percentage of nitrogen in the dry matter is also considerably lower. The yields of clover show a similar but less pronounced trend, and the N-percentage is not affected. This is in contrast to the results of Virtanen (1928) and

TABLE 4.

Influence of Reaction on Growth of Lucerne and Subterranean Clover in Sand Culture.

	pH of Sand.		Yield of Dry Matter, gm. per Pot.				% N in Dry Matter.	
			Tops.		Roots.			
	Initial.	Final.	Mean.	S.D.	Mean.	S.D.	Tops.	Roots.
Lucerne.	5·6–5·8	5·7–6·2	0·33	±0·13	0·13	±0·07	3·11	2·31
	6·0–6·4	5·7–6·1	1·40	±0·61	0·50	±0·33	4·17	2·70
	6·2–6·6	6·0–6·6	2·10	±0·60	0·96	±0·39	4·13	2·51
	7·4–7·7	6·8–7·1	2·51	±0·35	1·14	±0·32	4·34	2·50
Clover.	5·2–5·8	5·1–5·4	1·51	±0·45	0·28	±0·10	3·66	2·93
	6·1–6·3	5·5–5·6	3·81	±0·94	0·54	±0·19	3·58	2·93
	6·2–6·4	5·2–5·8	4·49	±0·82	0·58	±0·08	3·60	2·73
	7·4–7·7	6·3–6·6	5·40	±0·53	0·73	±0·12	3·61	3·01

Bjälfve (1934), who found that in other species of clover the N-percentage was lower at pH 5 than at pH 6. It is noteworthy that in all the clover-pots the reaction changed towards acidity during growth. The agreement between replicate pots is in both plants rather unsatisfactory, as regards both crop yields and pH-values. There was, however, a close correlation between the reaction and the crop yield of *individual* pots. The correlation coefficient between initial pH and total weight of dry matter (tops + roots) amounted to +0·718 in lucerne and +0·800 in clover.

The nodule formation in both plants was affected by the reaction in a very striking manner, as shown in Table 5: at acid reaction the nodules were bigger and fewer, at neutral to alkaline reaction smaller and more numerous. Many lucerne plants in the most acid sand had altogether failed to develop nodules, and several had died from nitrogen starvation at an early period, so that defective numbers of plants were left in these pots at the time of harvest; this may have rendered the crop yield at this reaction even smaller than it actually should be. The differences between nodule numbers are statistically highly significant, but the nitrogen percentage of the dry nodule-tissue is not markedly affected by the reaction, again in contrast to the results of Virtanen (1928) who found a consistent decrease in the nitrogen percentage of pea- and clover-nodules from sand of pH 6·0, 5·5 and 5·0. It thus appears that under the conditions of this experiment even moderate degrees of acidity, but especially pH-values between 5 and 6, have a strong inhibitory effect on the ability of both *Rh. Meliloti* and *Rh. Trifolii* to invade the plant roots and set up nodule formation. When viewed in comparison with the results obtained in agar cultures, this supports the contention of Bjälfve (1934), that sand treated with sulphuric acid is a somewhat unfavourable medium for inoculation experiments with legumes, and does not give results that can directly be applied to natural soils. No more experiments of this kind were therefore carried out, but instead plants were grown in naturally acid sand where the reaction was altered by means of small amounts of calcium carbonate or soil.

TABLE 5.

Influence of Reaction on Nodule Formation in Sand Culture.

	Lucerne.			Subterranean Clover.	
pH of sand	5·6–5·8	6·0–6·4	7·4–7·7	5·2–5·8	6·3–7·7
Plants examined	70	53	80	42	25
Nodules per plant*	1·0±0·24	7·1±0·85	22·6±1·41	10·9±1·45	43·2±2·81
Average weight of nodules, mgm.	6·6	2·6	1·2	3·33	1·94
% N in dry matter of nodules ..	7·8	7·8	8·0	7·35	7·44

* Mean and Standard Error (S.E.=S.D./$\sqrt{n}$).

A moderately fine river sand of pH approximately 5·9 was used in the subsequent experiments. A preliminary test was made with lucerne which was grown for 105 days (sown 17th August, 1942). The following fertilizer mixture was added per pot with 3 kgm. of sand: $CaHPO_4$ 0·5 gm., K_2HPO_4 and KH_2PO_4 each 0·1 gm., $MgSO_4$ 0·2 gm., NaCl 0·2 gm., and $FeCl_3$ 0·1 gm. Alkaline reaction was produced by means of 0·2% $CaCO_3$. Owing to bad germination the crop yields gave no significant results, but the numbers and size of nodules showed a similar response to pH as observed in the previous experiment:

	$-CaCO_3$	$+CaCO_3$
pH of sand	5·8–5·9	7·6–7·7
Number of plants examined	20	21
Nodules per plant, mean and standard error	13·1±1·50	36·8±4·18
Average weight of nodules, mgm.	4·1	1·3

A similar but more elaborate experiment was carried out with subterranean clover. Four replicate pots of each reaction were sown on 23rd March, 1943, and 12 seedlings were left per pot after germination. The plants were harvested after 118 days, when all nodules were removed from the roots and analysed separately. Table 6 shows the results.

TABLE 6.

Growth and Nodule Formation of Subterranean Clover in Acid and Alkaline Sand.

pH of sand initially		6·0		7·4
„ „ „ finally		4·8–4·9		7·4–7·7
Crop Yield.		Mean.	S.D.	Mean.	S.D.
Dry matter, gm., Tops		5·36	±0·537	5·34	±0·945
„ „ „ Roots		1·38	±0·318	1·35	±0·336
„ „ „ Nodules		0·33	±0·037	0·27	±0·044
Total nitrogen, mgm.		255·1	±30·5	216·2	±37·3
Percentage N in dry matter. Tops		3·48		2·82	
Roots		2·85		2·76	
Nodules		8·82		9·34	
Nodules per plant, mean and S.E.		153·0±8·61		98·9±7·00	
Average nodule weight, mgm.		1·32		1·54	
			t.		P.
Significance of differences (n=6)*	Nodules		3·714		0·02—0·01
	Total N.		1·593		0·2–0·1

* By Student's *t*-test (Fisher, 1936). Values of P less than 0·05 are taken as indicating significance.

The yield of dry matter in tops and roots is not affected by the reaction, but the weight of nodule-substance is significantly higher at acid reaction, where also the nitrogen percentage in the tops is increased, while that of the roots and nodules is only slightly affected. The difference in yield of total nitrogen, however, does not reach significance. The number of nodules is, in this experiment, quite appreciably reduced at alkaline reaction, a fact in agreement with the preference of *Rh. Trifolii* for a faintly acid reaction (Jensen, 1942). On the other hand, it forms a marked contrast to the results found in the previous clover-experiment (Table 5); this gives further evidence that the results depend to a large extent on the means by which the reaction is altered.

A similar experiment was designed to show the effect of a smaller change in reaction on the acid side (pH 5 to 6) on nodule formation. The same sand as used in the previous experiment was further acidified by addition of 4 per cent. of a heavy, humus-rich loam of pH 4·8; this gave the sand a pH-value of 5·3. Triplicate pots with pure sand and sand + soil (basal fertilizer as before) were sown with lucerne and subterranean clover on 23rd March, 1943; 12 seedlings of lucerne and 10 of clover were left per pot. Lucerne was harvested after 104 and clover after 90 days. The results are found in Table 7.

Both plants show a marked but rather irregular acidification of the medium during growth, a phenomenon which also appeared in the previous experiment with clover in sand without lime. Yet the influence of the initially different pH is quite striking in lucerne, which has given significantly higher yields of dry matter, both roots and tops, at the less acid reaction. The total weight of nodule-substance is not affected, but the nodules are again significantly fewer and bigger at the higher degree of acidity. The nitrogen percentage in tops and especially in nodules is, surprisingly enough, higher in the more acid medium; it appears that the metabolism of the plants has in some way been modified by some factor, possibly a minor element, added with the soil. The total yield of nitrogen, however, remains significantly higher at the less acid reaction. The yield and nitrogen percentages of clover are hardly affected by the reaction at all, except that the weight of nodule-substance is somewhat higher in the more acid medium; also

TABLE 7.

Growth and Nodule Formation of Lucerne and Subterranean Clover in Sand at Two Degrees of Acidity.

		Lucerne.		Clover.	
pH of sand initially		5·3	5·8	5·3	5·8
„ „ finally		5·0–5·6	4·4–5·6	4·9–5·0	4·9–5·2
Dry matter, gm., Tops	Mean	2·66	3·25	4·73	5·02
	S.D.	±0·284	±0·072	±0·401	±0·758
„ „ „ Roots	Mean	3·03	4·48	1·61	1·51
	S.D.	±0·526	±0·340	±0·402	±0·116
„ „ „ Nodules	Mean	0·22	0·21	0·16	0·13
	S.D.	±0·030	±0·041	±0·042	±0·029
Percentage N in dry matter ..	Tops	2·86	2·57	3·08	3·21
	Roots	1·60	1·61	2·74	2·74
	Nodules	8·25	5·80	9·01	8·33
Total N in plants, mgm. ..	Mean	142·2	167·7	202·8	213·2
	S.D.	±12·1	±8·54	±20·6	±28·0
Number of nodules per plant	Mean	17·2	38·9	46·7	44·9
	S.E.	±2·35	±3·35	±3·17	±3·42
Average weight of fresh nodules, mgm. ..		4·51	2·81	1·80	1·73
		t.	P.	*t.*	P.
Significance of differences (n=4).	Tops	3·481	0·05–0·02	0·584	0·6–0·5
	Roots	4·010	0·02–0·01	0·414	0·7–0·6
	Nodules	0·318	0·8–0·7	3·468	0·05–0·02
	Total N	2·976	0·05–0·02	0·506	0·7–0·6

the average number and size of nodules are the same at both reactions. Upon the whole it is noteworthy that the inhibitory effect of acidity is far less drastic in this medium than in the sand acidified with sulphuric acid.

Plants grown in soil.—Lucerne and subterranean clover were grown in a soil prepared by mixing 5 parts of river sand with one part of a heavy, acid loam very rich in organic matter. The resulting mixture had pH 5·0 and contained approximately 0·075% Total-N. Alkaline reaction was produced by addition of 0·2% lime, and 0·05% commercial superphosphate was given as a basal fertilizer. Four replicate pots with and without lime were sown with each plant species, and 12 seedlings were left per pot. Lucerne was sown on 10th March, 1943, and harvested after 124 days; clover was sown on 23rd March, 1943, and harvested after 138 days. The results are seen in Table 8.

The crop yield as well as the percentages of nitrogen in tops and roots of lucerne is very much (in total N almost 100%) higher at neutral to alkaline reaction, but the influence on the development of nodules is even more striking: at pH 4·9–5·1 the nodules are roughly nine times less numerous and on the average individually twelve times heavier than at pH 6·7–7·3, so that, within the limits of error, the same amount of nodular substance is produced at both reactions. The formation of big, branched, coral-like nodules in acid soil was more striking in this than in any of the other experiments. In this experiment alone there is also a really marked reduction in the nitrogen percentage of nodular substance at acid reaction. In clover, the yield of tops is not significantly affected, but the weight of roots and especially of nodules is considerably higher at acid reaction. Owing to the somewhat higher nitrogen percentage in tops at neutral reaction, however, the returns of total nitrogen are not significantly different. The average number of nodules is seen to be somewhat depressed and their individual size increased in the acid soil, but the influence of pH is very small in comparison with the effect on lucerne.

All the experiments recorded in Tables 6 to 8 thus show consistently that with increasing acidity the total yields of dry matter and nitrogen are either diminished or

TABLE 8.
Growth of Lucerne and Subterranean Clover in Acid and Alkaline Soil.

		Lucerne.		Clover.	
pH of soil, initially		4·9	6·7	4·9	6·7
„ „ finally		5·0–5·1	7·0–7·3	4·9–5·2	7·4–7·5
Dry matter, gm., Tops	Mean	1·79	3·27	4·17	3·69
	S.D.	±0·079	±0·389	±0·282	±0·460
„ „ „ Roots	Mean	1·86	2·45	1·34	0·99
	S.D.	±0·181	±0·379	±0·107	±0·172
„ „ „ Nodules	Mean	0·17	0·11	0·23	0·11
	S.D.	±0·048	±0·012	±0·017	±0·015
Percentage N in dry matter	Tops	3·63	4·33	2·98	3·29
	Roots	2·13	2·85	2·79	2·76
	Nodules	6·63	8·98	8·66	8·24
Total N in plants, mgm.	Mean	115·9	221·2	180·7	158·3
	S.D.	±7·53	±28·1	±12·1	±20·9
Number of nodules per plant	Mean	8·8	76·7	103·9	128·5
	S.E.	±0·66	±5·17	±4·37	±8·67
Average nodule weight, mgm.		9·67	0·82	1·35	0·62
		t.	P.	*t.*	P.
Significance of differences (n=6).	Tops	7·548	<0·01	1·786	0·2–0·1
	Roots	7·568	<0·01	3·422	0·02–0·01
	Nodules	2·384	0·1–0·05	7·980	<0·01
	Total N	12·53	<0·01	2·243	0·1–0·05

else not significantly influenced, but the weight of nodule-substance, if affected at all, is actually increased. This effect of the reaction stands out more clearly if the weight of nodule-substance is calculated as percentage of total dry matter. As shown in Table 9, this relative nodule-weight is in all cases significantly higher at lower pH. Consequently it appears that the nitrogen-fixing efficiency of the nodules, expressed as uptake of plant-nitrogen per unit weight of nodule-substance, decreases with increasing acidity; a significant influence of pH is seen in three of the five experiments summarized in Table 9. If we assume that the nitrogenous constituents of the nodules represent more accurately the nodule-fraction active in nitrogen fixation, and calculate the efficiency as ratio of total to nodule-nitrogen, we find that the efficiency increases with pH in all cases except in clover grown in moderately acid sand (Table 6).

It is scarcely necessary to say that these figures give us only an approximately true picture of the real efficiency of the nodule-tissue at different hydrogen ion concentrations, since they only represent the ratio between net uptake of nitrogen by the plants during their whole growth period, and weight of the nodules at the time of harvesting; the activity of the nodules, however, varies considerably according to the stage of development (Bond, 1936; Wilson, 1940). Moreover, no allowance is made for the possible excretion of nitrogenous compounds from the roots (although this is not very likely to take place under Australian conditions) or for uptake of combined nitrogen from the medium, which probably covered a good deal of the nitrogen supply of the plants in the soil experiment (Table 8). If these two items in the nitrogen economy of the plants are influenced by the reaction, the difference between the figures representing the true efficiency of the nodules may be considerably modified. It seems, however, that there can be no doubt about the general principle; for instance, in the experiment recorded in Table 7, the uptake of combined nitrogen is likely to be higher (and the net fixation consequently lower) in the more acid medium where a small quantity of soil was added to the sand; a correction for this uptake should then further reduce the efficiency of the nodules at the lower pH. However, a complete and accurate picture of the phenomena involved would first of all require periodical determinations of the

TABLE 9.

Relative Weight and Efficiency of Root Nodules at Different Reactions.

Plant and Medium.	pH. Initial.	Weight of Nodules in % of Total Dry Matter.		Uptake of N, mgm. per gm. Nodule Substance.		Ratio $\frac{\text{Total N}}{\text{Nodule-N}}$.	
		Mean.	P.	Mean.	P.	Mean.	P.
Lucerne ..	5·3	3·7		667		8·1	
Sand			0·05–0·02		0·1–0·05		<0·01
Table 7 ..	5·8	2·6		808		13·9	
Lucerne ..	4·9	4·4		728		10·6	
Soil			<0·01		<0·01		<0·01
Table 8 ..	6·7	1·9		2,041		22·6	
Clover ..	6·0	4·7		774		8·8	
Sand			0·05–0·02		0·7–0·6		0·8–0·7
Table 6 ..	7·4	3·9		798		8·6	
Clover ..	5·3	2·5		1,282		14·2	
Sand			<0·01		<0·01		<0·01
Table 7 ..	5·8	1·9		1,683		19·9	
Clover ..	4·9	4·0		806		9·3	
Soil			<0·01		<0·01		<0·01
Table 8 ..	6·7	2·4		1,380		16·6	

increases in total plant-nitrogen and weight of nodules, as in the experiments of Bond (1936) on the rate of fixation and transfer of nitrogen in soybeans. Secondly, and this is of particular importance in soil experiments, it would be necessary to determine the changes in nitrogen content of the medium, so that a complete balance-sheet of nitrogen can be constructed. Experiments in this direction are being continued.

The internal reaction of root- and nodule-tissue.—The apparently lower efficiency of the nodules at acid reaction may be due to several factors, among which may be mentioned the generally lessened uptake of mineral constituents of importance for the process of nitrogen fixation, such as molybdenum (Stephens and Oertel, 1943). Actually the plant material, which in another experiment (Jensen and Betty, 1943) had shown a striking difference in molybdenum content of lucerne nodules from soil of pH about 5 and 7·5, was produced in the same soil-sand mixture as the one used for the experiment recorded in Table 8. Another factor of obvious importance is the reaction of the root- and nodule-tissue itself. If this varies according to the reaction of the external medium, the latter might exert a profound influence on both the development and the activity of the nodules. The reaction of the root-juice in general is known to vary within limits narrower than those of the growth-substrate (Fred *et al.*, 1932). Comparatively wide variations were observed by Virtanen (1928), who found that the reaction of root-juice from clovers and peas grown in sand of pH 5 to 6 was very similar to that of the corresponding sand, but in more acid medium (pH 4·0–4·5) it was often 0·5–0·7 pH-units higher. On the other hand, Troug and Meacham (1919), Haas (1920) and Bjälfve (1934) found very little or no difference in the reaction of clover, lucerne and vetch roots taken from soil of different pH. Again it is interesting to note that these materials were taken from natural soil, but Virtanen's from artificially acidified sand. Only a few observations have yet been made on the reaction of the nodule-tissue itself. Robotnova (1936), using a micro-glass electrode, found internal pH-values of 5·8 to 7·1 in young nodules of pea, vetch and lupin, and Pietz (1938), who used vital staining of tissue-sections with indicator-dyes, confirmed by electrometric measurements, stated that the bacterial tissue in nodules of *Vicia faba* had a reaction in the neighbourhood of pH 6. Since these data give no information about the correlation between external

and intra-nodular pH, a number of determinations was made of pH in roots and detached nodules of various leguminous plants taken from soil, sand or agar medium of varying reaction. As soon as possible after collecting the plants, the roots were carefully washed, finally with distilled water, all nodules were picked off, the root-material was cut into small pieces with scissors and crushed in a mortar by means of a glass pestle, and the expressed fluid was used for pH-determination. The smaller roots from agar cultures were treated in a manner similar to the nodules, which were collected and washed on a filter of gauze, dried superficially with blotting-paper and then by means of a blunt glass rod crushed finely together with approximately their own weight of distilled water, thus forming a dense suspension which was used for the pH-determination. Addition of small amounts of dilute acid or alkali showed that the mixture had a very high buffer-capacity, so that little change in pH is likely to have resulted from the dilution of the nodule-content with water. The results are shown in Table 10.

TABLE 10.

Reaction of Root Nodules and Roots compared with Reaction of the Medium.

Plant.	Habitat.	pH.		
		Nodules.	Roots.	Medium.
Melilotus indica	Soil.	5·70	5·78	6·9
,, ,, ...	,,	5·80	5·26	7·2–7·3
Medicago denticulata	,,	5·60	5·50	5·8
,, ,, ...	,,	6·32	5·70	6·0
,, ,, ...	,,	6·06	5·82	6·8–7·0
,, ,, ...	,,	6·28	5·96	7·9
M. sativa	,, (Pot exp.).-	5·78		4·9–5·4
,, ,, ...	,, ,, ,,	5·90		7·5–8·0
Trifolium repens	,,	5·90	5·52	5·0
,, ,, ...	,,	5·55	5·82	6·2
,, ,, ...	,,	5·86	5·58	6·5
,, ,, ...	,,	5·84	5·60	7·0
,, ,, ...	,,	6·10	5·50	7·5
T. glomeratum	,,	5·72	5·52	6·1
,, ,, ...	,,	5·82	5·70	6·4
,, ,, ...	,,	5·94	5·70	6·6–7·2
T. subterraneum	,,	6·32	6·00	6·7
Lupinus angustifolius	,,	5·72		5·4
Vicia sativa	,,	6·12	5·68	6·3–6·9
Phaseolus vulgaris	,,	6·20	5·92	7·3
M. sativa	Sand (Pot exp.).	6·00		5·7–6·0
,, ,, ...	,, ,, ,,	5·90		6·0–6·1
,, ,, ...	,, ,, ,,	6·08		6·8–7·1
T. subterraneum	,, ,, ,,	5·96		5·1–5·4
,, ,, ...	,, ,, ,,	6·08		6·3–6·5
M. sativa	Agar culture	5·98	5·72	5·1
,, ,, ...	,, ,,	5·72		5·3–5·6
,, ,, ...	,, ,,	5·76	5·82	6·4
,, ,, ...	,, ,, (+ $CaCO_3$)	6·02	6·16	7·7–7·8
T. repens	,, ,,	5·70		5·3–5·5

Correlation coefficients between pH-values of:

	r	n	P
Nodules and medium	+0·342	28	0·1-0·05
Nodules and roots	+0·397	18	0·1-0·05
Roots and medium	+0·334	18	>0·1

The reaction of the nodules is seen to vary between pH 5·55 and 6·32 (mean, 5·92, standard deviation ± 0·214), which agrees well with the data of Pietz (1938) and partly also those of Robotnova (1936). The reaction of the actual root-tissue is slightly more

acid, from pH 5·26 to pH 6·16 (mean 5·72, standard deviation ± 0·209); the results are entirely similar to those found by Troug and Meacham (1919), Haas (1920) and Bjälfve (1934). The difference between pH of roots and corresponding nodules is significant by the *t*-Test ($t = 4{\cdot}332$, $n = 19$, $P < 0{\cdot}01$). Otherwise there are no significant correlations between pH-values of nodules, roots and external medium, although the last vary over an interval of nearly 3 pH-units. Neither does there appear to be any appreciable difference in the pH of nodules from different species. In the roots of young seedlings the reaction seems to some extent influenced by the medium, as shown by the following pH-determinations in crushed root-material of 14-days' old lucerne and clover seedlings grown in limed and unlimed acid soil, and in acid and alkaline agar medium:

Plant.	Nature of Medium.	pH of Medium.	pH of Roots.
T. subterraneum	Soil.	5·2	5·58–5·70
,, ,, ...	,,	7·8	6·10–6·14
,, ,, ...	Agar.	4·4–4·5	5·50–5·62
,, ,, ...	,,	7·5–7·8	6·00–6·12
M. sativa	,,	4·6–4·7	5·72
,, ,, ...	,,	7·6–7·9	6·12

Here the reaction of the root-tissues appears definitely to vary in the same direction as that of the growth substrate, but still it remains close to, or slightly below, pH 6. A reaction of this order also appears to be characteristic of the nodule-tissue, i.e., the substrate for the process of nitrogen fixation. (Incidentally this range of reaction is well below the optimum for growth of *Rh. Meliloti* in pure culture, but is approximately optimal for *Rh. Trifolii*, as previously determined (Jensen, 1942); this preference for acid reaction seems previously to have been observed only by Snieszko (1928), whose studies unfortunately did not include *Rh. Meliloti*.) It therefore appears that once the bacteria have entered the root-tissues the external medium has only little influence on them, especially in fully developed nodules where no correlation between internal and external pH is apparent.

Discussion.

The first conclusion that can be drawn from these experiments is that the reaction of the growth substrate appears to influence the growth of *Medicago* and *Trifolium* in N-free medium first and foremost by determining the number of root-nodules formed. This number is generally highest at neutral to faintly alkaline reaction, and decreases with increasing acidity; in clover it may also, as indicated by one experiment, decrease at only moderately alkaline reaction. The pH-limits for nodule formation are not only different in *Medicago* and *Trifolium*, but depend also greatly on the composition of the medium, presumably because this also regulates the survival of the bacteria and thus their entry into the roots. Sand acidified with sulphuric acid seems particularly unfavourable, while natural soil on the other hand permits nodule formation on lucerne at pH 4·9–5·0, which is well below the limit for growth of the rhizobia in pure culture; it remains uncertain whether this phenomenon is due to increased viability of the bacteria in soil, or merely to the soil being a heterogeneous mixture containing zones of locally different pH. The second explanation seems more likely, since some culture experiments gave no evidence that *Rh. Meliloti* was able to multiply in sterile soil of pH 5·2 (cf. Bryan, 1923*b*).

The influence of reaction on the growth and activity of the nodules appears to be of a different nature. When the numbers of nodules diminish at increasing acidity, the individual nodules tend to increase in size, presumably because a larger supply of nutrients becomes available to each nodule. This phenomenon seems previously to have been mentioned only by Sewell and Gainey (1930), who give no numerical data. This increase in size of individual nodules results in a relatively, and sometimes also actually, increased production of nodule-substance with increasing acidity. The efficiency of the nodules, expressed as uptake of plant-nitrogen per unit of nodule-matter or nodule-nitrogen, appears on the other hand to be inversely correlated with the hydrogen ion concentration. Also these processes of nodule development and activity depend largely on the composition of the medium, but always lucerne appears more

sensitive to acid reaction than subterranean clover, which may develop equally well at pH 5 and 7 (cf. Janssen, 1929).

Root nodules, when once formed, may continue to fix nitrogen at pH-values prohibitive to the formation of new nodules, owing to the fact that the internal reaction of the nodules as well as the root-tissues is but little influenced by the reaction of the surrounding medium. The decreased efficiency of the nodules in acid medium thus seems due to other causes than direct modification of the intra-nodular reaction, unless—and this possibility is by no means excluded—the biochemical mechanism of nitrogen fixation is so delicately poised that its functioning may be significantly influenced by changes in nodule-pH too small to be detected by the method with which the present data were obtained.

Summary.

Experiments on nodule formation on seedlings grown in agar medium of different reaction showed that a pH-range of 5·3 to 5·4 was critical for nodule formation on *Medicago*. In several species of *Trifolium*, nodule formation was seen at pH 4·5 and even pH 4·2. When once formed, nodules could continue to fix nitrogen at pH-values too low for formation of new nodules.

Lucerne (*M. sativa*) and subterranean clover (*T. subterraneum*) were grown in various sand and soil media within a pH-range of approximately 5 to 7·5. As a rule, fewer and bigger nodules were produced at increasing degree of acidity; this effect varied considerably in different experiments, but was upon the whole more pronounced in lucerne than in clover. Both plants formed nodules in natural soil at pH 4·9–5·0, while sand acidified with sulphuric acid seemed very unfavourable for nodule formation. The yield of lucerne was reduced at pH below 6, but the yield of clover was little affected. The dry matter yields and nitrogen percentages of the plants, as well as the numbers and size of nodules, seemed to depend not only on the pH-value, but also on the composition of the growth substrate. The relative weight of the nodule-substance was constantly, and the actual weight frequently, increased at acid reaction. Evidence was found that an inverse correlation exists between the nitrogen-fixing efficiency of the nodules and the hydrogen ion concentration of the growth substrate.

The internal reaction of root nodules was found to vary from pH 5·55 to pH 6·32, around a mean of pH 5·92. The root-tissues were upon the whole slightly more acid (pH 5·26–6·12, mean pH 5·72). No significant correlations were found between pH-values in nodules, roots and growth substrates, the last varying between pH approximately 5 and 8. In roots of young seedlings of lucerne and subterranean clover the reaction appeared to vary in the same direction as that of the growth substrate, but remained within the limits of pH 5·5 to 6·1. Generally it appears that the influence of soil reaction on nodule formation is different from that on the subsequent nitrogen-fixing activity, and that the latter is subject to wider pH-limits, i.e., the free-living rhizobia are more sensitive to acid reaction than the nitrogen-fixing complex of plant + rhizobia.

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References.

Almon, L., and Baldwin, I. L., 1933.—The Stability of Cultures of *Rhizobium*. *J. Bact.*, 26: 229-250.

Bjälfve, G., 1934.—Baljväxternas kväveupptagende och dess samband med jordens näringsinnehål och reaktion. *Medd. Centralanst. Försöksv. Jordbruksomr.*, No. 443 (Stockholm).

Bond, G., 1936.—Quantitative Observations on the Fixation and Transfer of Nitrogen in the Soya Bean (etc.). *Ann. Bot.*, 50: 559-578.

Bryan, O. C., 1923*a*.—Effect of Reaction on Growth, Nodule Formation and Calcium Content of Alfalfa, Alsike Clover and Red Clover. *Soil Sci.*, 15: 23-26.

————, 1923*b*.—Effect of Acid Soil on Nodule-forming Bacteria. Ibid., 15: 37-40.

Donald, C. M., 1941.—Pastures and Pasture Research. Sydney.

Fisher, R. A., 1936.—Statistical Methods for Research Workers. London, 6th Ed.

Fred, E. B., Baldwin, I. L., and McCoy, E., 1932.—Root Nodule Bacteria and Leguminous Plants. Madison, Wis.

Haas, A. R. C., 1920.—Studies on the Reaction of Plant Juices. *Soil Sci.*, 9: 341-369.

Hyland, H. L., 1938.—Comparison of Legume Growth in Different Soil Types of Varying Acidity Levels. *J. Amer. Soc. Agron.*, 30: 111-121.

Janssen, G., 1929.—The Comparative Acid Tolerance of Some Southern Legumes. *Soil Sci.*, 27: 469-496.

Jensen, H. L., 1942.—Nitrogen Fixation in Leguminous Plants. i. Proc. Linn. Soc. N.S.W., 67: 98-108.

————, and Betty, R. C., 1943.—Id., iii. Ibid., 68: 1-8.

Joffe, J. S., 1920.—The Influence of Soil Reaction on the Growth of Alfalfa. *Soil Sci.*, 10: 301-307.

Olsen, C., 1925.—Studies on the Growth of Some Danish Agricultural Plants in Soil with Different Concentration of Hydrogen Ions. *C. R. Trav. Lab. Carlsberg*, 16, No. 2.

Peterson, H. B., and Goodding, T. H., 1941.—The Geographic Distribution of *Azotobacter* and *Rhizobium meliloti* in Nebraska Soil in relation to Certain Environmental Factors. *Neb. Agric. Exp. Stat. Res. Bull.* 121.

Pietz, J., 1938.—Beitrag zur Physiologie des Wurzelknöllchenbakteriums. *Cent. Bakt.*, ii, 99: 1-32.

Powers, W. L., 1927.—The Effect of Hydrogen Ion Concentration on the Growth of Certain Plants. *Soil Sci.*, 24: 1-7.

Robotnova, I. L., 1936.—Okislitelno-voostanovitelnyi rezhim azotousvayatelei gruppy *Rhizobium*. *Mikrobiologiya*, 5: 217-239.

Sewell, M. C., and Gainey, P. L., 1930.—Interrelation of Nutrients and Soil Reaction on Growth and Inoculation of Alfalfa. *Soil Sci.*, 30: 297-305.

Snieszko, S., 1928.—L'influence exercée par la concentration des ions d'hydrogène du milieu nutritif sur le développement des bactéries des nodosités (etc.). *Bull. Int. Acad. Pol. Sci., Cl. Sci. Math. Nat.*, B, 55-74.

Stephens, C. G., and Oertel, A. C., 1943.—Responses of Plants to Molybdenum in Pot Experiments on the Cressy Shaley Clay-loam. *J. Counc. Sci. Indust. Res.*, Melbourne. 16: 69-73.

Troug, E., and Meacham, M. R., 1919.—Soil Acidity. ii: Its Relation to the Acidity of the Plant Juices. *Soil Sci.*, 7: 469-474.

Virtanen, A. I., 1928.—Über die Einwirkung der Bodenazidität auf das Wachstum und die Zusammensetzung der Leguminosenpflanzen. *Biochem. Z.*, 193: 300-312.

Watenpaugh, W. H., 1936.—The Influence of the Reaction of the Soil Strata upon the Root Development of Alfalfa. *Soil Sci.*, 41: 449-467.

Wilson, J. K., 1926*a*.—Effect on Nodulation of Supplementing the Legume Bacteria of the Soil with Artificial Cultures.—*J. Amer. Soc. Agron.*, 18: 280-294.

————, 1926*b*.—Legume Bacteria Population of the Soil. Ibid., 18: 911-919.

Wilson, P. W., 1940.—The Biochemistry of Symbiotic Nitrogen Fixation. Madison, Wis.